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Identification of Inhibitors of PLU-1, a Histone Demethylase that Promotes Breast Cancer Progression

PRINCIPAL INVESTIGATOR:

Raymond C. Trievel, Ph.D.

CONTRACTING ORGANIZATION:

University of Michigan
Ann Arbor, MI 48109-0606

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Raymond C. Trievel

Email: rtrievel@umich.edu

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14. ABSTRACT

PLU-1 is a member of the JARID1 family of Jumonji C histone demethylases and specifically demethylates the di- and trimethylated forms of Lys4 in histone H3 (H3K4me2/3). Demethylation of H3K4me2/3 by PLU-1 silences the expression of genes that regulate cell cycle progression in breast cells, promoting invasive cancer. These findings have been corroborated by mouse model studies demonstrating that the activity of PLU-1 is essential to cell proliferation, suggesting that PLU-1 inhibitors hold potential in treating breast cancer. The aim of this project is to express recombinant PLU-1 for use high-throughput screens (HTS) to identify small molecule inhibitors. Toward this goal, multiple constructs encompassing the catalytic domain of PLU-1 have been prepared and tested for soluble expression. The expression tests revealed that the PLU-1 constructs were poorly expressed or insoluble in bacteria. Based on these findings, an alternative approach was adopted wherein we tested the expression of RBP2, an H3K4me2/3-specific demethylase that is highly homologous to PLU-1. These tests demonstrated that most RBP2 constructs were not expressed or insoluble, with the exception of two constructs that showed limited solubility. These findings indicate that additional screening will yield soluble enzymes that can be used in HTS for PLU-1 inhibitors.

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INTRODUCTION

PLU-1 (also known as JARID1B and KDM5b) has been identified as an oncogene whose expression is upregulated in malignant breast cancers[1]. This protein is a member of the Fe(II)- and 2-oxoglutarate (2-OG)-dependent Jumonji C (JmjC) family of histone lysine demethylases and specifically demethylates the di- and trimethylated forms of Lys4 in histone H3 (H3K4me2/3)[2]. H3K4me3 is enriched in the promoter regions and 5′ coding regions of actively transcribed genes, whereas H3-K4 hypomethylation is associated with silent genes. Consistent with these findings, upregulation of PLU-1 represses the expression of genes that regulate cell cycle progression in breast cells, such as BRCA1, HOXA5, and CAV1[2, 3]. Correlatively, siRNA-mediated knockdown of PLU-1 has been shown to substantially reduce neoplasia in a mouse mammary tumor model[2]. More recently, PLU-1 has been shown to maintain cells in a proliferative state by silencing genes implicated in cellular differentiation and cell cycle control, including BMI1, EGR1, and p27[4]. Collectively, these findings define a direct relationship between the demethylase activity of PLU-1 and the proliferative capacity of invasive breast tumors, suggesting that PLU-1 inhibitors hold significant therapeutic potential in treating breast cancer.

BODY

PLU-1 is an oncogene that has been implicated in breast and prostate cancer[1-3]. This protein belongs to the JARID1 family of the JmjC class of histone lysine demethylases, which are Fe(II)-dependent hydroxylases that utilize the co-substrates 2-oxoglutarate and molecular oxygen[5, 6]. These enzymes catalyze lysine demethylation via a radical-based mechanism that oxidizes the methyl group to produce formaldehyde and the demethylated lysine residue[6]. PLU-1 specifically demethylates H3K4me2 and H3K4me3 to silence genes that regulate cancer cell proliferation[2-4]. The aim of this project is to express recombinant forms of PLU-1 for use *in vitro* high-throughput screens (HTS) to identify small molecules that inhibit its demethylase activity. Once PLU-1 is expressed and purified, a fluorescent demethylase assay that is commercially available will be used to screen a library of ~60,000 compounds for inhibitors.

The first phase of this project involves expressing constructs of the PLU-1 catalytic domain for subsequent purification and kinetic characterization. Twelve constructs of mouse PLU-1 were cloned as Mocr fusion proteins for small-scale expression and solubility screening in *E coli*. The Mocr protein is a newly engineered fusion tag of ~17 kDa in molecular weight that can effectively solubilize recombinant proteins comparable to GST and MBP tags[7]. The Mocr tag is designed as an N-terminal fusion in a pET-based vector and possesses a hexahistidine tag for affinity purification using a Ni or Co sepharose column. A tobacco etch virus (TEV) protease cleavage site has been engineered between the C-terminus of Mocr and the N-terminus of the passenger protein to permit removal of the Mocr tag during purification.

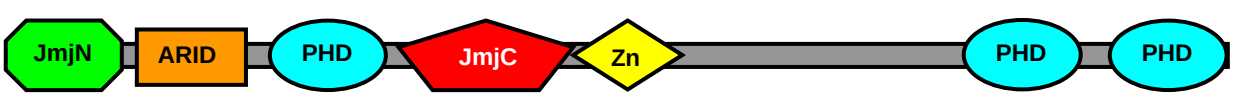

Figure 1: PLU-1 domain structure. PLU-1 is a 1544 amino acid polypeptide that is composed of multiple domains. The N-terminal half of the enzyme (1-751) possesses histone demethylase activity and is composed of five distinct domains: JmjN domain (residues 32-72), ARID domain (97-187), PHD domain (305-359), JmjC domain (453-619), and a C5HC2 Zn finger domain (692-744)[2]. The C-terminal region of Plu-1 contains two additional PHD domains that have been implicated in chromatin binding[8].

Constructs of PLU-1 were designed based on a bioinformatics analysis of its sequence, domain structure, and predicted secondary structure. The N-terminal half of the enzyme contains its JmjC catalytic domain as well as several smaller domains flanking the JmjC domain (Figure 1)[2]. A series of twelve constructs representing the N-terminal half of the enzyme and truncated proteins lacking the JmjN, ARID, and/or PHD domains were designed and cloned as Mocr fusion proteins (Figure 1 and Table 1). The vectors encoding the Mocr-PLU-1 fusions were transformed in Rosetta2 DE3 cells (Novagen) and induced overnight at 20 °C to optimize soluble expression of the recombinant proteins. The cells were harvested the following day and lysed using a commercial bacterial lysis reagent (Bugbuster, Novagen). Cells were lysed at pH values 6.5, 7.5, and 8.5 to account for the pI values of the Mocr-PLU-1 fusions when assessing protein solubility. The soluble and insoluble fractions of the cell lysate were separated by centrifugation, and the insoluble pellet was solubilized in 6 M urea buffer. The soluble and insoluble fractions were then resolved on an SDS-PAGE gel. A plant Rubisco methyltransferase, which has a molecular weight similar to the Mocr-PLU-1 fusion proteins, was used as a positive control for induction and solubility. A representative solubility test for two Mocr-PLU-1 fusion proteins is depicted in Figure 2.

Enzyme	Construct	Mocr Fusion Protein, Molecular Weight (kDa)	Expression Test at pH 6.5	Expression Test at pH 7.5	Expression Test at pH 8.5
PLU-1	30-760	100.9	No Expression	No Expression	No Expression
	30-772	102.3	No Expression	No Expression	No Expression
	30-791	104.5	No Expression	No Expression	No Expression
	77-760	95.5	No Expression	No Expression	No Expression
	77-772	96.8	No Expression	No Expression	No Expression
	77-791	99.1	Insoluble	Insoluble	Insoluble
	302-760	69.4	No Expression	No Expression	No Expression
	302-772	70.7	Insoluble	Insoluble	Insoluble
	302-791	72.9	Insoluble	Insoluble	Insoluble
	362-760	62.8	No Expression	No Expression	No Expression
	362-772	64.1	Insoluble	Insoluble	Insoluble
	362-791	66.4	Insoluble	Insoluble	Insoluble
RBP2	17-744	100.2	Insoluble	Insoluble	Insoluble
	17-756	101.6	No Expression	No Expression	No Expression
	17-775	103.9	No Expression	No Expression	No Expression
	64-744	94.8	Poor solubility	Poor solubility	Poor solubility
	64-756	96.1	Insoluble	Insoluble	Insoluble
	64-775	98.5	Insoluble	Insoluble	Insoluble
	286-744	69.5	Insoluble	Insoluble	Insoluble
	286-756	70.8	No Expression	No Expression	No Expression
	286-775	73.2	No Expression	No Expression	No Expression
	346-744	62.8	Insoluble	Insoluble	Insoluble
	346-756	64.1	Insoluble	Insoluble	Insoluble
	346-775	66.5	Poor solubility	Poor solubility	Poor solubility

Table 1: Summary of the results of the expression and solubility screens of the Mocr-PLU-1 and Mocr-RBP2 fusion proteins expressed in Rosetta2 DE3 cells and lysed at three different pH values.

The solubility tests revealed that seven of the Mocr-PLU-1 fusion proteins were not expressed at an appreciable level (Table 1). The remaining five constructs expressed well but were found in the insoluble fraction (Figure 2), indicating that the proteins formed inclusion bodies when expressed in the bacteria. Due to the poor expression and solubility of the PLU-1 constructs and the need to express large quantities of enzyme for HTS, we examined whether RBP2 (also known as JARID1A and KMT5A), a closely related homolog of PLU-1, could be expressed and purified to serve as a surrogate enzyme in HTS assays. A similar strategy has proven successful in identifying inhibitors of homologous histone methyltransferases using HTS[9]. RBP2 displays identical substrate specificity to PLU-1 in demethylating H3K4me2/3[10, 11], and its catalytic domain shares 65% sequence identity and 78% sequence conservation with

that of PLU-1. Twelve constructs encompassing the catalytic domain of human RBP2 were designed and cloned as Mocr fusion proteins as described above for PLU-1. Solubility tests of the Mocr-RBP2 fusions demonstrated that four of the constructs did not express, six were insoluble, and two showed poor solubility (Table 1). The solubility of these two constructs is insufficient for purification of the enzyme for kinetic characterization and subsequent HTS. However, these findings imply that additional screening of other PLU-1 homologs, such as the JARID1 H3K4me2/3-specific demethylases SMCX and SMCY[12-15], may yield sufficient quantities of soluble enzyme to enable HTS to identify JARID1-specific inhibitors.

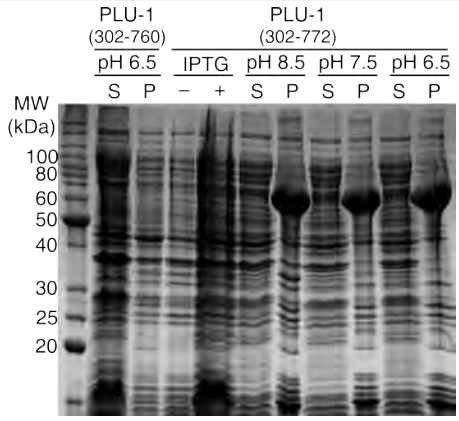

Figure 2: Representative solubility screens for the Mocr-PLU-1 (302-760) and Mocr-PLU-1 (302-772) fusion proteins resolved on a 12% SDS-PAGE gel. Lanes labeled with isopropylthiogalactoside (IPTG – and IPTG +) represent uninduced and induced bacterial samples, respectively. Each Mocr-PLU-1 was tested for solubility at three pH values using buffers containing TRIS pH 8.5, HEPES pH 7.5, or Bis-TRIS pH 6.5. The soluble supernatant (S) and the insoluble pellet (P) are shown for each solubility test.

KEY RESEARCH ACCOMPLISHMENTS

- Expression tests were performed for twelve constructs of the catalytic domain of PLU-1 that were expressed as Mocr-tagged fusions to enhance protein solubility.
- The Mocr-PLU-1 fusion proteins were not expressed at an appreciable level in bacteria or formed insoluble inclusion bodies at three different pH values.
- Additional solubility screens were conducted on twelve constructs of RBP2, a JARID1 H3K4me2/3-specific demethylase that is highly homologous to PLU-1.
- Solubility tests of the Mocr-RBP2 fusions demonstrated that two constructs displayed very limited solubility, whereas the remaining ten constructs did not express or were insoluble when tested under the same conditions as used for PLU-1.

REPORTABLE OUTCOMES

The data described in this report will be presented at the 2011 Era of Hope Conference in Orlando, Florida.

CONCLUSION

In the first phase of our project to screen for inhibitors of PLU-1, we tested the twelve constructs of the PLU-1 catalytic domain that had been cloned with a Mocr fusion tag for expression and purification from bacteria. The recombinant Mocr-PLU-1 fusion proteins did not express at a detectable level or were insoluble despite tests at different pH values. Due to the requirement to express large quantities of PLU-1 enzyme for HTS, an alternative approach was chosen in which we tested the expression of constructs of RBP2, a JARID1 demethylase that

shares high sequence homology with PLU-1. Expression and solubility tests of Mocr-RBP2 fusion proteins demonstrated that two constructs displayed very limited solubility, whereas the remaining ten constructs were either insoluble or did not express. Based on these results, additional construct screening of other PLU-1 homologs, including the JARID1 demethylases SMCX and SMCY, is currently being undertaken. We expect that these studies will provide soluble enzyme that can then be purified, kinetically characterized, and utilized in HTS for inhibitors. We envision that small molecules that inhibit PLU-1 demethylase activity may serve as lead compounds for the development of novel classes of drugs designed to treat metastatic breast cancer.

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